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Bio-tunable acoustic node assembly of organoids

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Many tissues in the human body are composed of densely-packed cells and minimal extracellular matrix (ECM). The resulting close cell proximity is important to retain cell viability and function^[1]. The ability to generate three-dimensional (3-D) tissues with high-cell packing density and predefined complex structures is of interest to mimic diverse tissues such as liver, cardiac or brain for therapeutics, diagnostics and drug screening applications^[2]. The controlled assembly and organization of multi-cellular systems to mimic native tissues is critical to tissue engineering. Many technologies have been proposed to engineer 3-D tissue constructs with control over geometry and spatial cell distribution. Most of these existing technologies are based on assembling cell encapsulating microscale hydrogels^[3], scaffold seeding techniques^[4] and extrusion/laser-based bioprinting^[5]. These scaffold-based techniques are useful for wide applications such as gene delivery^[6], bone regeneration^[7] and cell differentiation^[8]. However, these methods cannot provide physiologically-relevant cell-packing density and control over the cell proximity for bioengineering tissues with low ECM. Scaffold-free tissue engineering focuses on generating highly organized cell constructs, promoting direct cell-cell interactions and interfering with autologous ECM alignment. In this context, spheroid assembly is a recently introduced a strategy of scaffold-free tissue engineering, which provides the most viable method to build larger tissue constructs^[9]. Cell spheroids are spherical cell aggregates that

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Additional information

P. C. and U. D. are inventors on a US Patent (pending) related to the technology in this manuscript. UD is a founder of and has an equity interest in, DxNow, a company that is developing microfluidic and imaging technologies for point-of care diagnostic solutions. UD's interests were reviewed and are managed by the Brigham and Women's Hospital and Partners HealthCare in accordance with their conflict of interest policies.

provide the highest possible initial cell packing density for bottom-up tissue engineering and are therefore useful as 3-D building blocks for scaffold-free assemblies. Spheroid assembly technologies include spheroid printing^[10] via micronozzles and magnetic cell spheroid assembly^[11], which requires loading magnetic nanoparticles into cell spheroids. While spheroid printing can produce highly complex tissue constructs, it suffers from long assembly time that prohibits building large, centimeter-scale, constructs. On the other hand, magnetic approach provides rapid assembly for building large structures but it is severely limited by the geometry of tissue structures -dictated by the geometry of the magnet- it can produce and potential adverse effects of magnetic nanoparticles on cell physiology and metabolism^[12].

In this study, we introduce a bio-tunable acoustic node assembly technique for bioengineering of heterogeneous multicellular architectures from cell spheroids in a scaffold-free fashion. This technique enables the assembly of large number of cell spheroids ($>10^4$) within a few seconds to form unique complex structures in a fluidic environment beneath the nodal regions of acoustic surface standing waves. Generated 3-D assembly can be dynamically tuned to diverse patterns by altering the wave frequency. Acoustic node assembly technique maintains the viability and functionality of the assembled cell spheroids, which spontaneously merge into a single organoid over several days of culture. Using this technique, we first generate homogenous/heterogeneous tissue constructs from fibroblast and human umbilical vein cell (HUVEC) spheroids to investigate the cell spheroid fusion and formation of large-scale constructs. Furthermore we assembled long-term viable hepatic constructs from primary rat hepatocytes, fibroblast and HUVECs; and evaluate these constructs for formation of bile canaliculi, hepatic gap junctions and extracellular matrix. This versatile method is useful for a variety of 3-D tissue engineering applications such as 3-D tumor models for personalized medicine, stem cell and developmental biology and microphysiological systems for drug screening.

We explore acoustic surface standing waves, here Faraday waves, and their hydrodynamic affect at the bottom of a chamber^[13, 14], as a contactless and cytocompatible tool to assemble large amounts of cell spheroids in a fluidic environment. Faraday waves are generated by vertical vibration of a liquid layer at the acceleration amplitude^[15]. Faraday waves take diverse waveforms as their basic unit ranging from square, circle, stripe to highly complex geometries, which are determined by fluid properties (*i.e.*, surface tension, viscosity), geometrical boundary conditions as well as vibrational parameters (*i.e.*, frequency and acceleration)^[16]. The feature size of these waveforms is determined by the wavelength of the standing waves that can be described by the inviscid dispersion equation as below^[17],

$$(\lambda f)^2 = \left[\frac{g\lambda}{2\pi} + \frac{\sigma}{\rho_l} \left(\frac{2\pi}{\lambda} \right) \right] \tanh\left(\frac{2\pi H}{\lambda} \right) \quad (1)$$

where f is the Faraday wave frequency; λ is the Faraday wavelength; g is the gravitational acceleration constant; σ is the surface tension; ρ_l is the liquid density; and H is the thickness of the liquid layer in the assembly chamber. Based on this equation, feature size of the standing waves can be tuned by altering the vibrational frequency. Vertical vibration of the

air-liquid interface generates a velocity field inside the liquid layer, which further creates a hydrodynamic drag force on microscale objects on the substrate of the assembly chamber^[13, 14]. Under the low Reynold number condition, the hydrodynamic drag force (F_d) can be described by Stokes' law as below,

$$F_d = 6\pi\eta r v \quad (2)$$

where η is the dynamic viscosity, r is the radius of the particle, and v is the flow velocity relative to the particle. Microscale cell spheroids are driven by the hydrodynamic drag force to the regions under nodes of the standing waves, where amplitude of the standing waves is zero. Assembly patterns of cell spheroids can be predicted by the corresponding wave functions^[18].

One of the significant challenges in spheroid bioprinting technologies is high-throughput assembly^[19]. We assemble a large number of cell spheroids ($>10^4$) into a densely-packed structure using standing-wave induced hydrodynamic drag force in the fluid^[13, 20]. First, cell spheroids are generated from corresponding cell types in a low adhesion culture plates (Figure 1a and S1). Spheroids are suspended in a cell culture medium and loaded into an assembly chamber that is mounted on a vertical vibration exciter (Figure S2). Spheroids sediment and distribute randomly on the substrate. By applying standing waves, hydrodynamic drag force is generated due to the formation of the velocity field induced by periodic deformation of the air-liquid surface (Figure 1b). Cell spheroids on the substrate are driven to the regions beneath the nodal lines of the standing waves, while empty space is left on the region beneath the antinodes (Figure 1d). The assembly time ranges from a few seconds up to tens of seconds, which is determined by vibration acceleration. To retrieve the assembled construct we performed the acoustic node assembly in a fibrinogen and thrombin solution. Assembled construct was immobilized in fibrin hydrogel after crosslinking and further cultured for organoid formation (Figure 1c).

We have loaded 2.5×10^4 fibroblast spheroids into 4 cm^2 square-shaped assembly chamber with 1.6 mm depth and assembled them in periodical pattern. Assembly of randomly distributed spheroids starts with formation of standing waves. The spheroids are accumulated and densely-packed at the region beneath the node line of standing waves, forming a predefined pattern. The entire assembly process takes 12 sec (Figure 2a). When the vibration acceleration is too large, the assembled structure fails to form due to the formation of vortices and chaotic waves. After the standing waves are stopped the assembled pattern remains stable and intact due to the vertical supporting from substrate. Using acoustic node assembly, we can generate numerous versatile geometries and patterns in a predictive manner. We demonstrate diverse complex patterns of assembled spheroids generated by surface standing waves under various waveforms, harmonic order and symmetry modes (Figure 2b). The assembly pattern can be dynamically altered by tuning vibrational frequency and acceleration. A representative example of the reconfiguration process is demonstrated in Video S1. Randomly distributed samples are first assembled into ring shapes at 129 Hz. After tuning vibrational frequency to 120 Hz, the pattern transform into network shapes. After restoring frequency to the original value, the pattern transform into ring shapes again. Tunability of acoustic node assembly provides a facile way to create

complex 3-D tissue construct without changing the mold/template. To evaluate the effect of spheroid loading density on assemblies, we patterned spheroids in stripe geometry with loading densities ranging from 400 to 1300 spheroid cm^{-2} (Figure 2c). The line width in the assembled structure is determined by the number of loaded cell spheroids in the assembly chamber. At low loading densities (400 spheroid cm^{-2}), spheroids are individually aligned along nodal lines. As the number of loaded spheroids increases, the line width of assembled structure increases. The relation between the assembled line width and the number of spheroid exhibits a linear relation with $R^2 = 0.998$ (Figure S3).

We evaluated the cytocompatibility of the acoustic node assembly method in terms of cell viability and proliferation capacity. The assembled fibroblastic cell spheroids immobilized within fibrin hydrogel were cultured up to 72 hours post assembly and stained with Calcein AM for viable cells and with ethidium homodimer for dead cells (Figure 3a). The viability of cells were not affected by the assembly process as more than 90% of the spheroids are viable. To determine whether cells resume their proliferative behavior following the assembly process, fibroblast and HUVEC cell spheroids were immunostained with proliferation specific cell surface marker anti-Ki67 (Figure 3b–c). The cellular filapodial extensions and high expression of Ki67 suggest the preservation of metabolic activities of multiple cell types after acoustic node assembly in 3-D microenvironments.

Fusion of the cell spheroids is a critical biophysical process in bottom-up tissue engineering resulting in formation of large and complex organoids mimicking native tissues with low ECM^[21]. Spheroid fusion can be theoretically explained by differential adhesion hypothesis^[22]. In practice, interplays among contacted spheroids, and between spheroid and its surrounding matrix, are among the major driving forces in tissue fusion. We demonstrate dense spheroid packing using acoustic waves and analyze cell spheroid fusion dynamics up to 72 hours in the generated constructs composed of fibroblast and GFP-HUVEC spheroids (Figure 3d–f and Figure S4). The acoustic node assembly results in high cell spheroid packing density and the fusion of fibroblast, HUVEC and fibroblast-HUVEC spheroids is significant after 16 hours and is fully achieved in 72 hours. We also successfully demonstrate the fusion of cell spheroids from different cell types (*i.e.*, fibroblast spheroids and GFP-HUVEC spheroids) (Figure 3f). At early stages (16 h), fusion starts between adjacent spheroids. After 72 hours due to the cell growth and migration all cell spheroids (fibroblast and HUVEC) are fused, forming a merged tissue construct that is more homogenously distributed in cellular content (Figure 3f).

We evaluated the capacity of acoustic node assembly platform to generate functional tissue-engineered constructs. First, we assembled fibroblast spheroids alone and assessed their functionality at day 3 by immunostaining for collagen type 1, characteristic extracellular matrix (ECM) protein, which assist the stabilization of cells and form cellular niche (Figure 4a). Assembled fibroblast spheroids homogenously express collagen type I all over the fused construct at day 3 post-assembly. A demonstration of assembled 3D fibroblast construct is shown in Video S2. Further, we assembled GFP-HUVEC spheroids and characterized for the secretion of vascular basal lamina proteins. We immunostained constructs at day 3 for the endothelial cell marker CD31 and laminin (Figure 4b). All cells were CD31 positive and at the core of assembled spheroids, we detected accumulation of laminin, demonstrating the

functional elements of endothelial cell ECM. Next, we demonstrate assembly of different spheroids by mixing HUVEC and fibroblast spheroids in 1:1 ratio and characterize the vascularization behavior. These spheroids from two different cell types are distributed randomly without any selective accumulation (Figure 4c). HUVEC and fibroblast spheroids fuse within each other, where endothelial sprouts start to merge endothelial spheroids and form network like patterns (Figure 4e–f). The sprouting behavior is higher in co-culture conditions compared to HUVEC alone conditions, however the vascular basal membrane proteins (Collagen type IV) are similarly expressed selectively on the HUVEC spheroids. These findings strongly suggest that acoustic node assembly of the HUVEC spheroids in co-culture with fibroblast spheroids can be used as a pre-vascularization strategy.

We further evaluated the developed acoustic node assembly technology in engineering hepatic organoids. Histologically, liver tissue is highly specific in terms of structural composition. Only 3% of total liver tissue consists of extracellular matrix, where the rest of the organ is composed of cells. Currently, most of the methods developed for *in vitro* formation of liver tissue utilize scaffolds for cell seeding^[23]. Bioengineering scaffold-free liver tissue *in vitro* is essential to fabricate models to study hepatic diseases and generate drug toxicity testing platforms^[24]. Cell morphology, cell-to-cell contact and cell polarization are vital for *in vitro* hepatocyte culture that defines and regulates their cellular functionality^[25]. We utilized the acoustic surface standing waves for scaffold-free assembly of densely-packed hepatic tissue constructs. Accordingly, we have investigated the establishment of gap junctions between adjacent cells in the engineered liver tissue by staining anti-Connexin32 (Figure 5a). The analysis indicates that after 6 days of culture the cell-cell interactions are formed uniformly throughout our liver tissue. An important feature of hepatocytes is their capacity to remodel their microenvironment and secrete collagen type IV, their specific extracellular matrix (ECM). The immunocytochemistry analysis of extended culture (day 13) of assembled hepatic spheroids demonstrates that the hepatic spheroids are positive for collagen type IV. This indicates the maintenance of the ECM secretion capacity of assembled hepatic spheroids. Liver hepatic acini excrete the metabolites and toxic waste from the body through the bile canaliculi network^[26]. This function can be assessed in *in vitro* models structurally by a CMFDA transport assay, which provides qualitative information about cellular polarization of hepatocytes and the integrity of their transport capacity^[27]. In functional hepatocyte spheroids CMFDA diffuses into the cells and is hydrolyzed by enzymatic esterase reactions, which turns it in fluorescein and later excreted by bile canaliculi. We observed that after 6 days of culture following the assembly our liver tissues show clear formation of bile canaliculi at the hepatic junctions in the intercellular space (Figure 5b). A complementary analysis to CMFDA assay is the expression of multidrug resistance protein 2 (MRP2) on the apical side of hepatocyte plasma membrane, along the canalicular space. MRP2 is a canalicular organic anions transporter that facilitates the excretion of various drugs through biliary system^[28]. Our immunohistochemistry analysis indeed shows that MRP2 is well expressed in our liver tissue constructs in a network like fashion similar to the bile canalicular network (Figure 5c).

Conclusions

In this study, we propose a scaffold-free 3-D cell spheroid assembly method for bottom-up tissue engineering. The developed cytocompatible assembly method has capability to rapidly assemble thousands of spheroids in couple seconds in predefined patterns and generate functional organoids. The demonstrated acoustic node assembly method we can be utilized to generate pre-vascularized endothelial networks supporting the survival of the tissue-engineered constructs. Moreover, we demonstrate an *in vitro* model to generate 3-D hepatic constructs. This technology can be potentially applied to diverse tissue engineering applications as well as model platforms for *in vitro* toxicity studies.

Experimental Section

Experimental setup

The experimental setup for cell spheroid assembly is shown in Figure S2. We design a 20×20×1.55 mm chamber for acoustic node assembly (Figure S2 insert). The assembly chamber is fabricated from poly(methyl methacrylate) (PMMA) plates, double-sided adhesive (DSA) (iTapestore, Scotch Plains, NJ, USA) using a laser cutter (VersaLaser™, Scottsdale, AZ, USA) and mounted on the top of the vibration exciter (U56001, 3B Scientific, Tucker, GA, USA) via an banana-type adapter. The vibration exciter is stabilized on a metric tilt platform (Edmund Optics, NJ, USA) and leveled horizontally. The metric tilt platform is fixed to a vibration isolation pad (Grainger, CA, USA) to isolate mechanical perturbation. The vibration exciter is electrically driven by an audio power amplifier (Lepai LP-2020A+, Parts Express, OH, USA) and an arbitrary function generator (33510B, Agilent, CA, USA). Vertical vibration of the exciter is characterized by an accelerometer (MMA7341L, Freescale Semiconductor, TX, USA).

Primary rat hepatocyte isolation

Primary rat hepatocytes were freshly isolated from adult female Lewis rats (Charles River Laboratories, Massachusetts) weighing 150–200 g, according to modified procedure as described previously^[29]. All animals were treated in accordance with the National Research Council guidelines. The Subcommittee on Research Animal Care, Committee on Research at the Massachusetts General Hospital, approved the experimental protocols that relates to the handling and use of animals (cell isolation) in this manuscript (Permit # 2011N000111). We obtained approximately 2–3×10⁸ hepatocytes with >99% purity and 90–95% viability evaluated by trypan blue staining.

Spheroid formation

NIH 3T3 mouse fibroblast cells cultured in DMEM supplemented with 10% (v/v) FBS, 1% penicillin and streptomycin were harvested and plated in 60 mm low adhesion Petri dishes at 1×10⁶ cells/dish for 2 days. Green Fluorescein Protein (GFP)-Human umbilical vein endothelial cells (HUVEC) were monolayer expanded on gelatin coated (0.2% w/v) tissue culture plates in EBM supplemented with EGM-2 bullet kit (Lonza). Cells were harvested with Trypsin-EDTA (0.05%) and plated in 60 mm low adhesion Petri dishes at 1×10⁶ cells/dish for 2 days for the formation of HUVEC spheroids. Hepatocyte spheroids were

generated from freshly isolated rat hepatocytes directly plated in 60 mm low adhesion Petri dishes at 1.6×10^6 cells/dish for 2 days. Formed cell spheroids were collected and used in acoustic node assembly.

Generation of tissue constructs with acoustic node assembly

Cell spheroids were resuspended in 10 mg mL^{-1} human fibrinogen (Sigma) prepared in PBS and loaded on cover slip ($18 \times 18 \text{ mm}$) placed at the bottom of the assembly chamber. Before starting the acoustic node assembly $150 \text{ }\mu\text{L}$ of thrombin (Sigma) was added at 0.5 IU mL^{-1} final concentration and mixed homogeneously. After the sedimentation of cell spheroids, acoustic surface standing waves have been applied at $100 - 140 \text{ Hz}$ frequency and $1.2 - 1.8 \text{ g}$ amplitude ranges, resulting in formation of various assembly patterns. Acoustic surface standing waves were terminated at $t = 15 \text{ s}$. The immobilization (crosslinking of the fibrin) of assembled structures in formed fibrin hydrogel has been achieved after 20 min at room temperature. Constructs were recovered on cover slip and cultured in corresponding cell culture media at $5\% \text{ CO}_2$ and 37°C at humidified atmosphere.

Cell viability and proliferation assays

Cell viability test was evaluated with LIVE/DEAD[®] viability/cytotoxicity kit (L-3224, Molecular Probes). The LIVE/DEAD[®] staining solution was prepared by mixing $20 \text{ }\mu\text{L}$ ethidium homodimer and $5 \text{ }\mu\text{L}$ Calcein AM in the in 10 mL PBS. Constructs were incubated for $20 - 30 \text{ min}$ in staining solution at 37°C in humidified incubator and immediately imaged with fluorescent microscope (Zeiss, AxioVision).

Assessing Hepatocyte Polarization

Polarization and formation of bile canalicular network of hepatocytes in assembled constructs were evaluated at day 6 with CellTracker[™] Green CMFDA ($2 \text{ }\mu\text{M}$) (Life Technologies) in a Live Cell Imaging Solution (Life Technologies)^[24]. Constructs were immersed in CMFDA solution for $10 - 15 \text{ min}$ at 37°C in humidified incubator and immediately analyzed with fluorescent microscope (Zeiss AxioVision).

Tissue culture

Assembled cell spheroids were cultured at $5\% \text{ CO}_2$ and 37°C at humidified incubator in culture media according to cell types. Fibroblast cell spheroids were cultured in DMEM supplemented with $10\% \text{ FBS}$, $1\% \text{ penicillin-streptomycin}$ (Life Technologies), 0.1 TIU mL^{-1} Aprotinin (Sigma). Constructs with HUVECs were cultured in EBM supplemented with EGM-2 bullet kit (Lonza) and 0.1 TIU mL^{-1} Aprotinin. Tissue constructs generated from co-culture of fibroblast and HUVEC spheroids were cultured in 1:1 mixture of corresponding culture media. Hepatic constructs were cultured in serum-free Williams E medium supplemented with 0.05 U L^{-1} insulin, $0.01428 \text{ mg L}^{-1}$ glucagon, 20 ng mL^{-1} epidermal growth factor (EGF), $7.5 \text{ }\mu\text{g mL}^{-1}$ hydrocortisone, 200 U mL^{-1} penicillin, and $200 \text{ }\mu\text{g mL}^{-1}$ streptomycin at 37°C in humidified $10\% \text{ CO}_2$ environment.

Immunocytochemistry

Fibrin hydrogels containing assembled tissue spheroids were fixed with 4% paraformaldehyde for 20 min at room temperature and washed with excessive PBS. Hydrogels were permeabilized with 0.3% Triton-X 100 (Sigma) and blocked with 1% BSA (Sigma). Spheroids were stained overnight at 4°C for primary antibodies: anti-collagen type 1, anti-collagen type 4, anti-CD31 (Abcam), anti-laminin, anti-Ki67 (Abcam), anti-connexin 32 (Abcam) and anti-MRP-2. Samples were washed and stained 2 h at room temperature for secondary antibodies donkey anti-mouse Alexa Fluor® 488, donkey anti-rabbit Alexa Fluor® 568 and donkey anti-chicken Alexa Fluor® 647 antibodies (Jackson Immuno Research). Cytoskeleton was stained with phalloidin Alexa Fluor® 647 and DAPI was used as nuclear staining. Immunostained constructs were analyzed with confocal microscopy (Zeiss, LSM 710).

Data analysis and simulation

The images were processed using the ImageJ (NIH, Bethesda, MD). The data was analyzed and plotted using OriginPro™ (OriginLab, MA) software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Illustration of acoustic node assembly of organoids

a, Schematic of formation of cell spheroids on a low-adhesion surface. Cells are plated on a low-adhesion surface. Spheroids are formed after 2 days of incubation. **b**, Image of formed hepatocyte spheroids in 48 hours. **c**, Schematic demonstration of acoustic node assembly of cell spheroids. Cell spheroids are first loaded in the assembly chamber. Then standing waves are applied to assemble spheroids into predefined patterns. **d**, Demonstration of node and antinode zones on standing waves in a fluidic environment. **e**, Assembled spheroids are immobilized in fibrin hydrogel and matured in culture media. Adjacent spheroids are fused forming larger organoid. **f**, Fluorescence image of fused endothelial tissue construct from GFP-HUVEC spheroids.

Figure 2. Characterization of acoustic node assembly

a, Dynamic process of spheroid assembly. Randomly dispersed cell spheroids at 0 s start to form predefined assembly patterns at 3 s and final assembly at 12 s. Bright regions at $t=3$ s and $t=9$ s are light reflections by surface standing waves. **b**, Diverse assembly patterns from fibroblast spheroids. Assembly patterns were formed under different vibrational accelerations and frequencies. **c**, Effect of cell spheroid loading density on assemblies. Spheroid loading density is varied from 700 to 3700 spheroids cm^{-2} . Increasing the spheroid density increases the thickness of the stripes in the assembly motif. All scale bars indicate 2 mm.

Figure 3. Cytocompatibility of spheroid assembly process

a. Cell viability assay at t=0 and 72 hours after spheroid assembly. Live cells stained with Calcein AM (green) and dead cells stained for EthD-1 (red). **b–c,** Cell proliferation in assembled organoids demonstrated with anti-Ki67 (red) immunostaining at day 3. **b,** fibroblast tissue construct, **c,** Tissue construct formed from GFP-HUVEC spheroids. Cell cytoskeleton and nuclei shown with phalloidin (cyan) and DAPI (blue) respectively. **Fusion of cell spheroids. d.** Fibroblast spheroids, **e.** GFP-HUVEC spheroids and **f.** 1:1 mixture of fibroblast and GFP-HUVEC spheroids were assembled separately. Spheroid fusion was

recorded at $t=0$, 16, 48 and 72 hours. Individual cell spheroids at 0 h gradually start to fuse at 16 h. At $t=72$ h spheroids at all conditions are totally fused. Images do not represent identical zones over time. Scale bars: a) 100 μm , b) 20 μm c) 40 μm , d–f) 100 μm .

Figure 4. Functional analysis of assembled organoids

a, Assembled fibroblast spheroids start to synthesize extracellular matrix positive for anti-collagen type I (red) at day 3. Image insert shows magnified region, scale bar: 500 μm . **b**, Fragment of GFP-HUVEC (green) assembly showing secretion of vascular basal lamina stained for anti-laminin (red) at day 3 post assembly. **c**, Endothelial characteristics of endothelial organoids was confirmed with anti-CD31 staining (red), anti-laminin (cyan) positive nodules were indicated with white arrows. **d**, Co-culture of fibroblast and GFP-HUVECs are homogeneously assembled in a ring shape at day 3. Fibroblast zones are stained only for DAPI (blue). **e**, Magnified zone of fused fibroblast - GFP-HUVEC (green) organoid. Vascular basal lamina is localized within HUVEC zone and express anti-Collagen type IV (red). **f**, Magnified zone of **e** showing the angiogenic sprouting of endothelial cells (green), which demonstrate the initial phases of capillary formation in 3-D. Scale bars: a, b, c and e) 200 μm , d) 1mm and f) 100 μm .

Figure 5. Characterization of hepatic organoids

a, Fusion of hepatocyte spheroids forming cellular gap junctions at day 13 demonstrated with immunostaining of anti-connexin 32 (green). Secretion of hepatic extracellular matrix positive for collagen type IV (red) and cell cytoskeleton (cyan) indicate the integrity of assembled hepatic construct. **b**, Polarization of hepatocytes in assembled hepatic organoid showed formation of bile canaliculi (green) via CMFDA staining. **c**, Expression of MRP2 on cellular membrane (red) along the bile canaliculi network and co-localization of connexin 32 positive hepatocyte cell junctions (green) and cell cytoskeleton (white). Scale bars a and b) 100 μm c) 50 μm .